

The *Staphylococcus aureus* Autoinducer-2 Synthase LuxS Is Regulated by Ser/Thr Phosphorylation[▽]

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The *Staphylococcus aureus* autoinducer-2 (AI-2) producer protein LuxS is phosphorylated by the Ser/Thr kinase Stk1 at a unique position, Thr14. The enzymatic activity of the phosphorylated isoform of LuxS was abrogated compared to that of nonphosphorylated LuxS, thus providing the first evidence of an AI-2-producing enzyme regulated by phosphorylation and demonstrating that *S. aureus* possesses an original and specific system for controlling AI-2 synthesis.

The latest discoveries in the field of microbiology have proven that bacteria communicate between each other. In fact, many bacteria secrete small, diffusible signaling molecules. It is generally assumed that these molecules are used for a process termed “quorum sensing,” the phenomenon whereby the accumulation of specific, diffusible, low-molecular-weight signal molecules (or “autoinducers”) enables bacteria to sense when the minimal number, or “quorum,” of bacteria for a concerted response to be initiated has been achieved (22). Gram-positive and Gram-negative bacteria use quorum-sensing communication circuits to regulate a diverse array of physiological activities, like symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (22).

The only presently known quorum-sensing mechanism that appears to be shared by both Gram-positive and Gram-negative bacteria is based on a group of interconvertible, diffusible molecules collectively referred to as autoinducer-2 (AI-2). The LuxS protein required for AI-2 production (19, 26) is a metal-containing enzyme (29) that cleaves *S*-ribosyl-L-homocysteine (SRH) to generate homocysteine and the AI-2 precursor, 4,5-dihydroxy-2,3-pentanedione (DPD). Outside the cell, unstable DPD undergoes chemical rearrangement that converts the molecule to AI-2, a small molecule able to penetrate membranes and to diffuse in the medium, allowing cross-species quorum sensing.

The LuxS/AI-2 system in *Staphylococcus aureus*, a highly adaptable Gram-positive bacterium responsible for numerous clinical infections, has been analyzed in detail (13, 14, 28). Moreover, the emergence of antibiotic resistance has become a serious concern, especially due to methicillin-resistant *S. aureus* (MRSA) isolates that are resistant to all available penicillins and other β -lactam antimicrobial drugs (7). One appealing approach to this problem is to target bacterial systems associated with virulence mechanisms, particularly those based on signal transduction. Signal sensing leading to cellular re-

sponses must be tightly regulated to allow survival under variable conditions. The prevalent signaling mechanism in prokaryotes works through two-component systems. However, studies of the genomes of various pathogens have revealed a large family of eukaryotic-like Ser/Thr protein kinases (STPKs). It is becoming clear that signaling through Ser/Thr phosphorylation is a critical regulatory mechanism in pathogenic bacteria (16, 25). However, our understanding of *S. aureus* kinase biology has been seriously hampered by failure to identify relevant kinase substrates.

While there is a significant body of published work defining the molecular mechanisms by which bacterial cells communicate, little is currently known about the regulation of the LuxS enzymes. The present study was undertaken to determine if the AI-2 producer protein LuxS might be regulated posttranslationally via STPK-dependent mechanisms.

Stk1-mediated phosphorylation of *S. aureus* LuxS. The *S. aureus* genome encodes one Ser/Thr protein kinase, named Stk1 or PknB (8, 10). Stk1 appears to be involved in different key pathways, like those for cell wall metabolism, antibiotic susceptibility, and regulation of virulence (3, 9, 10, 20, 21). However, while the *S. aureus* STPK has been characterized, little is known about the nature of the corresponding substrates, and understanding of the biological role of this enzyme remains limited. A recent host-induced proteome study by Yuan et al. (27) listed the LuxS protein from *Bifidobacterium longum* as being potentially regulated via phosphorylation. Therefore, due to our interest in Ser/Thr kinase regulation in *S. aureus*, we decided to investigate whether the *S. aureus* AI-2 producer protein LuxS was regulated by Ser/Thr phosphorylation.

In order to establish whether the LuxS protein undergoes posttranslational modification by phosphorylation, we first investigated *in vitro*, in the presence of the purified Stk1 kinase, whether LuxS could be phosphorylated. Stk1 was expressed as a His-tagged fusion protein and purified from *Escherichia coli* as described previously (9). Recombinant LuxS was expressed and purified from *E. coli* BL21(DE3)Star, which harbors the pETPhos_LuxS plasmid (Table 1). *In vitro* phosphorylation was performed with 4 μ g of LuxS in 20 μ l of buffer P (25 mM

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> BL21(DE3)Star	F2 <i>ompT hsdS_B(r_{B2}⁻ m_{B2}⁻) gal dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Stratagene
Δ <i>stk1</i>	<i>S. aureus</i> ST1004 strain corresponding to an in-frame deletion of the Stk1 kinase	8
<i>E. coli</i> 10G	<i>E. coli</i> derivative; ultracompetent cells; F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74 endA1 recA1 araD139 Δ(<i>ara leu</i>)7697 <i>galU galK rpsL nupG-tonA</i>; used for general cloning</i>	Lucigen
pET28a	pET28a vector used for general cloning	Novagen
pMK4_Pprot	pMK4 shuttle vector; <i>E. coli</i> and <i>S. aureus</i> derivative with a constitutive promoter	1
pETPhos	pET15b (Novagen) derivative that includes replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site and Ser-to-Gly mutagenesis in the Nterm His tag	4
pCDFDuet-1	pET vector derivative designed for coexpression of two proteins under T7 <i>lac</i> promoter induction	Novagen
pCDFDDuet_Stk1/LuxS	pET vector derivative used for coexpression of Stk1 and LuxS proteins under T7 <i>lac</i> promoter induction	Novagen
pETPhos_LuxS	pETPhos derivative used to express His-tagged fusion of LuxS	This work
pETPhos_LuxS_T14A	pETPhos derivative used to express His-tagged fusion of LuxS_T14A	This work
pETPhos_LuxS_T14D	pETPhos derivative used to express His-tagged fusion of LuxS_T14D	This work
pMK4_Pprot_LuxS	pMK4_Pprot derivative used to express His-tagged fusion of LuxS in <i>S. aureus</i>	This work

Tris-HCl [pH 7.5], 1 mM dithiothreitol [DTT], 2.5 mM MgCl₂, 2.5 mM MnCl₂, and 1 mM EDTA) with 200 μ Ci/ml of [γ -³³P] ATP, corresponding to 65 nM (3,000 Ci/mmol; PerkinElmer), and 0.5 μ g of kinase for 30 min at 37°C. The proteins were separated by SDS-PAGE, and phosphorylation was analyzed by autoradiography. The presence of an intense radioactive signal indicated that LuxS was phosphorylated by Stk1 (Fig. 1A). As expected, no radioactive band was observed in the assay in the absence of Stk1. These data indicate that LuxS interacts with Stk1, suggesting that this protein is regulated in

S. aureus by extracellular signals mediated via the Ser/Thr kinase Stk1.

In vivo phosphorylation of LuxS. To assess the relevance of *in vitro* phosphorylation, the *in vivo* phosphorylation of LuxS in *S. aureus* was also investigated. To determine whether phosphorylation of LuxS occurs *in vivo*, Western blot analysis was performed using specific antiphosphothreonine, antiphosphoserine, or antiphosphotyrosine antibodies. To overproduce and purify phosphorylated LuxS, a His tag was attached at the C terminus of the LuxS protein under the constitutive Pprot

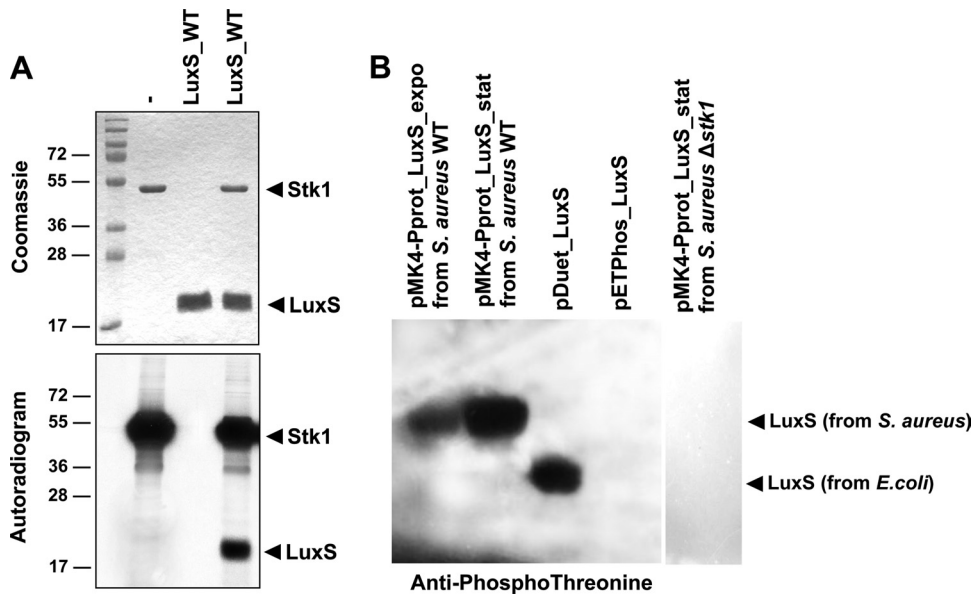


FIG. 1. (A) *In vitro* phosphorylation of LuxS by *S. aureus* Stk1. The recombinant Stk1 kinase encoded by the *S. aureus* genome was expressed and purified as a His-tagged fusion in *E. coli* and incubated with the purified His-tagged *S. aureus* LuxS. The different proteins were incubated together with [γ -³³P]ATP for 25 min, subjected to gel electrophoresis, and stained with Coomassie blue (upper panel). Radioactive bands were revealed by autoradiography (lower panel). Standard proteins of known molecular masses were run in parallel (far left lane). (B) *In vivo* phosphorylation of LuxS. Recombinant LuxS proteins purified from either *E. coli* or *S. aureus* strains were analyzed by SDS-PAGE and detected by immunoblotting, using antiphosphothreonine antibodies. The different profiles of migration of LuxS from *E. coli* and LuxS from *S. aureus* are due to the variation in length of the His tag in the two recombinant proteins.

TABLE 2. Primers used in this study

Primer	Gene	5' to 3' Sequence (restriction site) ^a
Nterm-pET28a-Pfs	<i>pfs</i>	GGGGGATCCATGATTGGTATAATTGGTGCCATGGAAGAAG (BamHI)
Cterm-pET28a-Pfs	<i>pfs</i>	GGGAAGCTTTTATAATTGAGACACTAATGCTTCAACAGTTTG ACT TG (HindIII)
Nterm-pETPhos-LuxS	<i>luxS</i>	TAATAGCTCCATGGATGACAAAAATGAATGTTGAAAGT (NdeI)
Cterm-pETPhos-LuxS	<i>luxS</i>	TATGGATCCTT4GTGATGATGATGATGATGCATATGTTTTCTGTACCGAAAAAC ATCATGCCATTC (BamHI)
Nterm-pMK4-LuxS	<i>luxS</i>	TAATAGCTGGATCCATGACAAAAATGAATGTTGAAAG (BamHI)
Cterm-pMK4-LuxS	<i>luxS</i>	TATCTGCAGTTAGTGATGATGATGATGATGCAATGTTTCCTGTACC (PstI)
Nterm-pETPhos-LuxST14A	<i>luxS(T14A)</i>	TAATAGCTCCATGGATGACAAAAATGAATGTTGAAAGTTTCAATTTAGATCAT GCTAAAGTG (NdeI)
Nterm-pETPhos-LuxST14D	<i>luxS(T14D)</i>	TAATAGCTCCATGGATGACAAAAATGAATGTTGAAAGTTTCAATTTAGATCAT GACAAAGTGGTTGCCCC (NdeI)

^a Restriction sites are underlined and are named in parentheses. Mutagenized codons are shown in bold.

promoter in plasmid pMK4-Pprot, allowing constitutive expression in *S. aureus* as previously described (1, 8) (Table 1). Cultures of *E. coli*_pETPhos_LuxS or *S. aureus*_pMK4-Pprot_LuxS overexpressing the His-tagged LuxS protein were collected and lysed, and the soluble LuxS was then purified to homogeneity using Ni-NTA agarose as described by the manufacturer (Qiagen). As shown in Fig. 1B, LuxS purified from the *S. aureus* strain was phosphorylated on threonine residues, thus establishing that the LuxS protein is phosphorylated *in vivo* and specifically on threonines, while no bands could be detected with antiphosphoserine or antiphosphotyrosine antibodies (data not shown). In contrast, the antiphosphothreonine antibodies did not react with recombinant LuxS protein purified from *E. coli*, thus confirming its specific phosphorylation in *S. aureus* (Fig. 1B). In order to check the specificity of the antibodies for the phosphorylated isoform, we used purified protein from the pDuet_LuxS strain as a control. Generation of the LuxS phosphorylated isoform was based on coexpression of the Stk1 kinase and its substrate, LuxS, in *E. coli*, using a strategy recently described by Molle et al. (17). The Stk1 kinase domain and wild-type LuxS (LuxS_WT) were cloned into the pCDF-Duet vector (Tables 1 and 2) and overexpressed; therefore, His-tagged, phosphorylated LuxS was purified as previously described, while the Stk1 kinase was not tagged and was therefore not copurified. As shown in Fig. 1B, we could specifically detect the phosphorylated LuxS protein produced via the Duet system, thus confirming that the antibodies recognized specific phosphorylated isoforms. Moreover, we confirmed the direct phosphorylation of LuxS by Stk1 *in vivo*. Using the same strategy, we transformed the pMK4-pProt_LuxS vector into the Stk1 null mutant strain (*Δstk1*) described previously by Débarbouillé et al. (8) and overexpressed and purified the LuxS protein from this strain devoid of the Stk1 kinase. As shown in Fig. 1B, we could not detect any phosphorylated LuxS isoform when LuxS was overexpressed in the *S. aureus* *Δstk1* strain, thus confirming that LuxS is a substrate of Stk1 *in vivo*.

LuxS is phosphorylated on a single threonine residue. To identify the number and nature of the phosphorylation sites, we opted for a mass spectrometry (MS) analysis, which was successfully developed previously (6, 11, 23, 24). To this aim, LuxS was incubated with cold ATP in the presence of Stk1 and subjected to mass spectrometry analysis after tryptic digestion. The sequence coverage of the protein was complete, and phos-

phorylation occurred on only one peptide (4–22), as illustrated by the tandem mass spectrometry (MS-MS) spectra, unambiguously confirming the presence of one phosphate group on Thr14 (Fig. 2A). This finding was consistent with our *in vivo* phosphorylation profile indicating that threonine residue(s) are phosphoacceptors.

Definitive identification and localization of Thr14 as the only phosphorylation site was achieved by site-directed mutagenesis to introduce a single mutation that prevents specific phosphorylation (Thr-to-Ala replacement), generating the LuxS phosphoablative mutant. This LuxS_T14A mutant was expressed, purified as a His-tagged protein in *E. coli* BL21(DE3)Star, which harbors plasmid pETPhos_luxS_T14A, and used in an *in vitro* kinase assay with [γ -³³P]ATP and Stk1. Equal amounts of proteins were separated by SDS-PAGE and analyzed by autoradiography (Fig. 2B). As expected, phosphorylation of the phosphoablative mutant was completely abrogated compared to phosphorylation of LuxS_WT, thus indicating that LuxS is phosphorylated only on the Thr14 residue. This finding was further supported by an additional round of mass spectrometry on the LuxS_T14A mutant pretreated with ATP and Stk1, which failed to identify any additional phosphate group that could eventually have arisen as a mechanism to compensate for the loss of the phosphorylation site (data not shown).

Localization of the Thr14 residue in the three-dimensional structure of LuxS. Multiple-sequence alignments show that LuxS is a highly conserved protein present in various bacterial species (Fig. 3A). The structures of several species have been determined by X-ray crystallography, but none is available as yet for *S. aureus*. However, we mapped its sequence on the structure of *Helicobacter pylori* LuxS, which is 68% identical to *S. aureus* LuxS. LuxS is a homodimer (Fig. 3B), and its active site contains a Zn²⁺ ion located at the interface between the monomers. The critical active-site Cys residue is located in the loop connecting helices α 2 and α 3. The threonine at position 14 (shown in bold) is part of a conserved sequence motif (LIVDHTKRVM) (Fig. 3A) located in a surface loop between the N-terminal 3_{10} helix and strand β 1. In the context of the homodimer, residues in this loop form noncovalent interactions with the α 2/ α 3 loop of the opposing monomer, with Thr14 positioned within approximately 12 Å of the active-site cysteine. Although not strictly invariant, Thr14 is highly conserved across both Gram-positive and Gram-negative bacterial species, including pathogenic and nonpathogenic organisms.

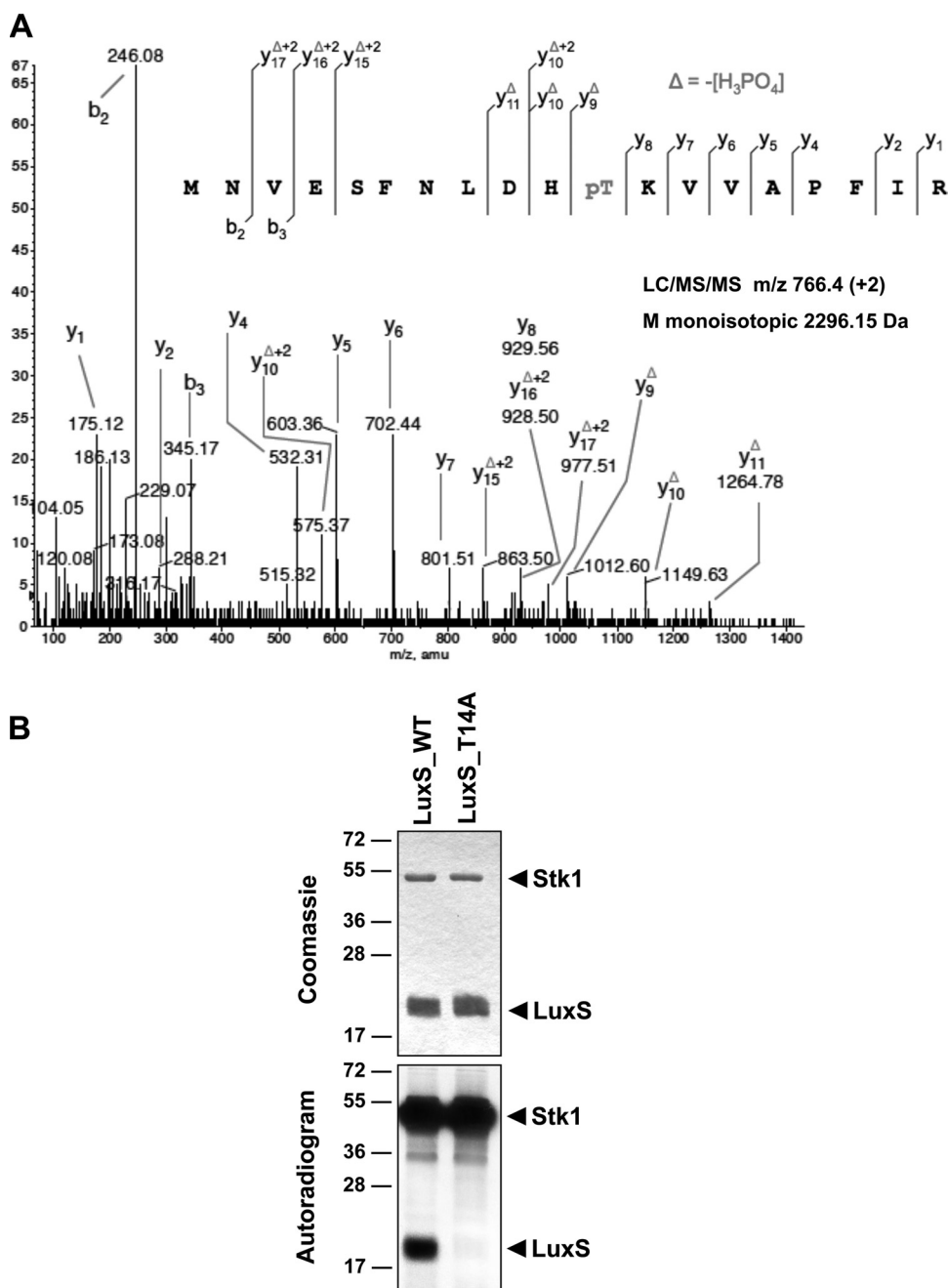


FIG. 2. (A) Identification of the LuxS phosphorylation site *in vitro*. MS-MS spectrum of the monophosphorylated peptide (4 to 22) at m/z 766.4 (+2) (monoisotopic mass, 2,296.15 Da). The unambiguous location of the phosphate group on threonine 14 is shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions lose phosphoric acid (−98 Da) after the threonine phosphorylated residue. (B) *In vitro* phosphorylation of the LuxS_T14A mutant. The purified LuxS_WT strain and LuxS_T14A mutant were incubated with Stk1 and [γ - 33]ATP. Samples were separated by SDS-PAGE (upper panel) and visualized by autoradiography (lower panel).

The mechanistic consequences of phosphorylation on Thr14 cannot be predicted unequivocally. However, this residue is flanked by the side chains of the invariant Asp12 and the conserved basic residue (Lys or Arg) at position 15. Phosphorylation of Thr14 would markedly affect the mutual interactions among these three side chains, as it is likely to affect the interaction with the $\alpha 2/\alpha 3$ loop and, therefore, in an indirect fashion, the active site. Moreover, it was recently shown that in

H. pylori, the highly conserved histidine residue adjacent to Thr14 (Fig. 3A) is critical for stabilization of the LuxS substrate (SRH) via a 3-Å hydrogen bond, as when mutated LuxS activity was reduced (2). Therefore, phosphorylation of Thr14, inducing a charge modification of the dimer surface, could disturb the histidine environment and thus influence SRH stabilization. Consequently, given that a small subset of LuxS orthologues does not feature a Thr or Ser at position 14, one

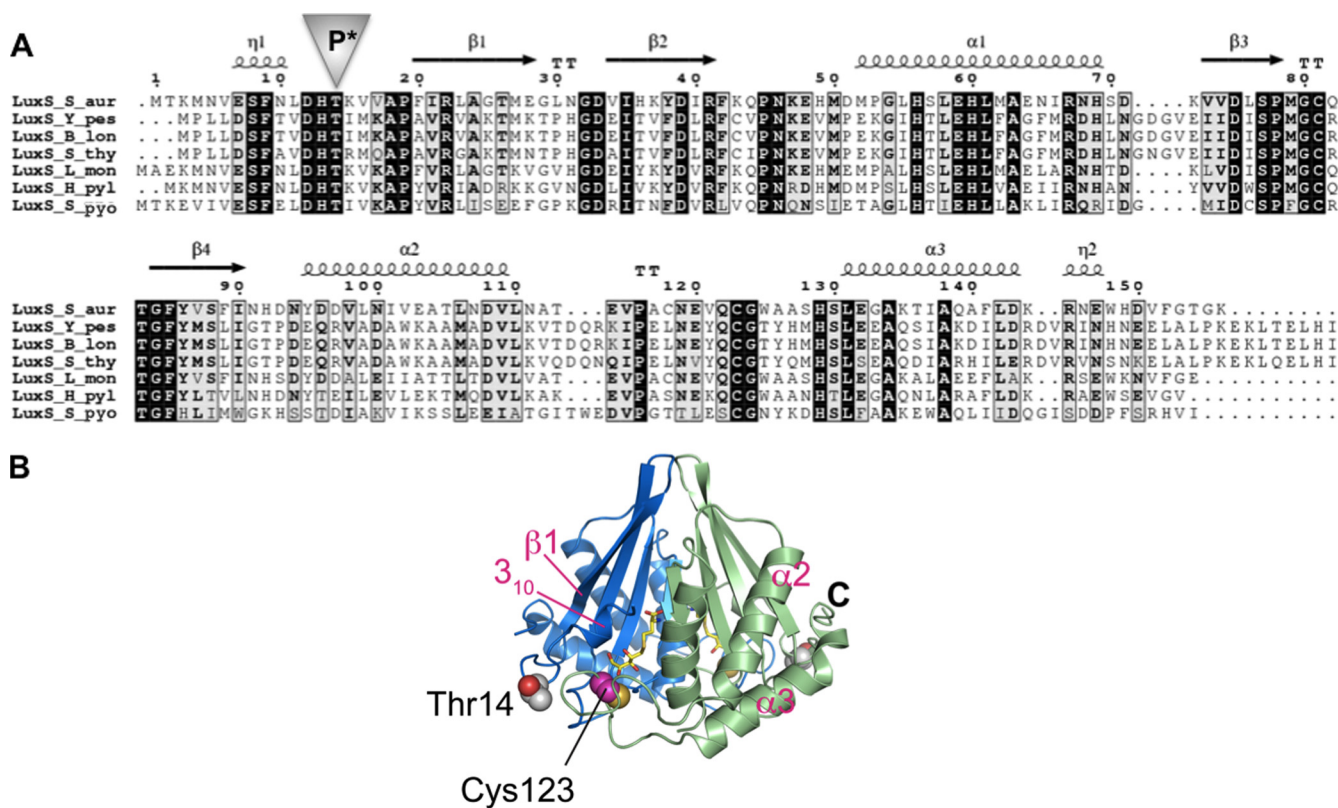


FIG. 3. (A) Conservation of the phosphoacceptor in LuxS orthologues. Multiple-sequence alignment of LuxS orthologues was performed using ClustalW and ESPript (*S_aur*, *Staphylococcus aureus*; *Y_pes*, *Yersinia pestis*; *B_lon*, *Bifidobacterium longum*; *S_thy*, *Salmonella enterica* serovar Typhimurium; *L_mon*, *Listeria monocytogenes*; *H_pyl*, *Helicobacter pylori*; *S_pyo*, *Streptococcus pyogenes*). Protein secondary-element assignments for LuxS are represented on the tops of the sequences. Numbering of the amino acids corresponds to that of the LuxS protein from *S. aureus*. Residues conserved in all species are presented in black boxes. The phosphorylated site of LuxS from *S. aureus* and its position are indicated by the large arrowhead. (B) Ribbon diagram of *H. pylori* LuxS (Protein Data Bank [PDB] entry 1j6x) (15). The positions of the putative Thr phosphorylation site and the active-site cysteine are highlighted by van der Waals spheres in gray and magenta, respectively. The stick model represents the catalytic 2-ketone intermediate in the structure of *B. subtilis* LuxS (PDB entry 1ycl) (18). Residues are labeled with respect to the amino acid sequence of *S. aureus* LuxS.

would predict that as a potential regulatory mechanism the phosphorylation of Thr14 would be detrimental to activity.

Phosphorylation abolishes LuxS activity. As revealed by the mapping on the LuxS structure, the Thr14 site appears critical for LuxS enzymatic activity. Therefore, following a strategy that has been successfully used to demonstrate that regulation of a substrate protein via phosphorylation is important during active cell growth in mycobacteria (Wag31) (12) or for mycolic acid metabolism (mtFabH or MabA) (23, 24), we generated the phosphorylation mimic LuxS_T14D by site-directed mutagenesis (Tables 1 and 2) and expressed and purified it. Previous studies have shown that acidic residues such as Asp or Glu qualitatively recapitulate the effect of phosphorylation with regard to functional activity (12, 23, 24). The option was taken to analyze and compare the activities of the LuxS_WT, LuxS_T14A, and LuxS_T14D strains.

In order to assess the effect of LuxS phosphorylation on its activity, we took advantage of the fact that LuxS also plays a metabolic role in the activated-methyl cycle (AMC). AI-2 is the adduct of borate and a ribose derivative and is produced from *S*-adenosylhomocysteine (SAH) (29). SAH is the by-product of numerous transmethylation reactions involving *S*-adenosylme-

thionine (SAM) (5). Hydrolysis of SAH by the nucleosidase Pfs yields *S*-ribosyl-L-homocysteine (SRH) and adenine (Fig. 4A). SRH is then converted to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) by *S*-ribosylhomocysteinase (LuxS) (29). DPD spontaneously cyclizes to form a furanone, which is complexed with borate to form AI-2. Each test to assess LuxS activity was prepared as described by Zhu et al. (29). Briefly, SRH was prepared by incubating SAH with the nucleosidase Pfs for 1 h at room temperature, and the completion of the reaction was monitored spectrophotometrically using the difference in absorption between SAH and adenine. The reaction was initiated by the addition of LuxS and monitored continuously at 412 nm in a spectrophotometer at room temperature. Surprisingly, neither of the LuxS mutants (T14A and T14D), was able to show any enzymatic activity, while the LuxS_WT strain generated the expected activity profile (data not shown). These results indicate that the Thr14 residue is critical for LuxS activity, as the Ala replacement was not able to restore LuxS activity as expected. In order to bypass this problem, we decided to compare the activity of the LuxS protein purified from the pETPhos_LuxS strain, which corresponds to the non-phosphorylated isoform of LuxS, with that of p-LuxS, which

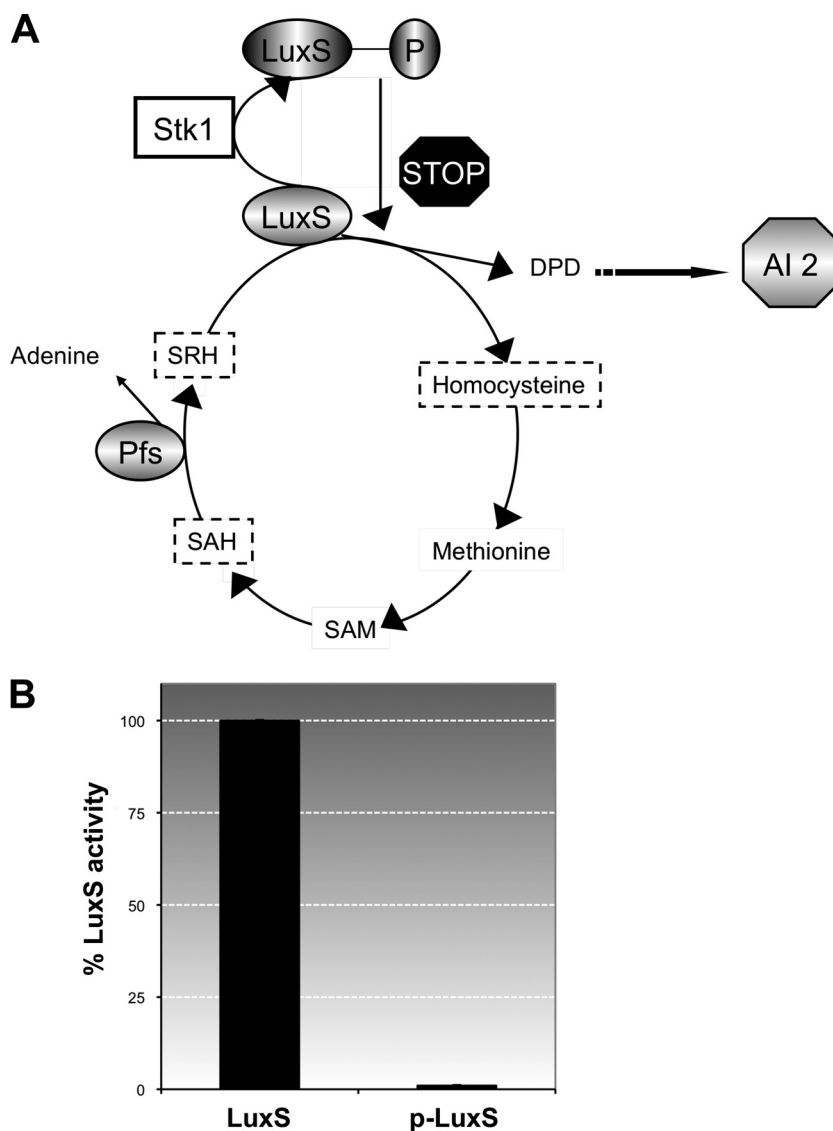


FIG. 4. (A) Scheme of AI-2 synthesis. The activated methyl cycle (AMC) drives the formation of methionine and its subsequent conversion to *S*-adenosylmethionine (SAM), which is primarily used for the methylation of DNA, RNA, proteins, and certain metabolites. Donation of the SAM methyl group leads to formation of the toxic metabolite *S*-adenosylhomocysteine (SAH). SAH is removed by one of two mechanisms involving either one (SAHh) or two (Pfs and LuxS) enzymes to generate homocysteine and complete the AMC cycle. The Pfs/LuxS pathway also leads to the generation of DPD, which spontaneously cyclizes to generate the furanones that constitute AI-2. (B) Comparative enzymatic activities of phosphorylated LuxS and nonphosphorylated LuxS. Wild-type LuxS proteins were purified either under nonphosphorylated isoforms (pETPhos_LuxS) or under phosphorylated isoforms (p-LuxS; pCDE-Duet_LuxS_Stk1), and LuxS activity was assayed. Two independent protein preparations were assayed in triplicate, yielding similar results.

corresponds to the LuxS phosphorylated isoform purified from the pETDuet system. The phosphorylation state of the p-LuxS protein was confirmed by mass spectrometry prior to the assay and indicated that 95% of the protein was phosphorylated on the Thr14 residue. As shown in Fig. 4B, activity of phosphorylated LuxS (p-LuxS) was almost totally abrogated compared to the activity of nonphosphorylated LuxS, indicating that the Stk1-mediated phosphorylation of LuxS seems to represent a key mechanism for regulating and/or controlling its activity. Phosphorylation seems to modulate LuxS activity with a rather strict on/off mechanism. However, further work is needed to

understand at a molecular level whether and how phosphorylation of LuxS modifies the overall structure of this protein.

In conclusion, although LuxS proteins have been extensively studied, no data were available with respect to their potential posttranslational regulation. We reported here, for the first time, the phosphorylation of the *S. aureus* AI-2 producer protein LuxS and identified Thr14 as the critical phosphoacceptor. This study suggests that environmental signals could trigger LuxS phosphorylation and presumably influence and/or regulate biological activities of the protein. Therefore, our findings may have important consequences for participating in and/or

regulating the biological activities assigned to LuxS and Stk1, especially in quorum sensing and consequently in *S. aureus* virulence. Whether phosphorylation at position 14, which is highly conserved in the LuxS protein family, contributes to the regulation of these homologues is an attractive hypothesis that remains to be addressed.

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